Sequencing refractory GC rich regions in plasmid DNA

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Those modifications of Sanger's DNA sequencing method which in principle allow for sequence analysis of any double stranded DNA ("plasmid sequencing") follow a three step protocol: *first,* restriction of the plasmid DNA downstream from the region to be sequenced; *second,* denaturation of the double stranded DNA by heat or alkali in the presence of an excess of oligonucleotide primer; *third,* rapid cooling or neutralization. However, when using the published procedures we were confronted with sequences refractory to this kind of sequence determination. We could, for example, not obtain sequence reference ladders from short DNA stretches with a Z-DNA forming potential (like $[CG/GC]_7$ and $[CA/GT]_7$) as well as from G/C rich repeated motifs (as in the 21 bp repeat of SV40) which we needed for mapping experiments. In the method presented here we *first,* render the DNA region relevant for primer annealing and sequencing single stranded, *second,* anneal a 5'-end [^{32}P]-labelled primer, and *third,* perform a Sanger sequencing reaction without a radioactively labelled dNTP. This allows for efficient primer annealing as well as for optimal dNTP concentrations. The only additional steps compared to published procedures are labelling of the primer and incubation with T4-DNA polymerase.

1. Primer labelling: 40 µl reaction volume containing 1 µg primer (150 pmole), 300 µCi [γ^{32}P]-ATP (100 pmol, 3000 Ci/mmole), 10 mM $MgCl_2$, 15 mM 2-mercaptoethanol, 50 mM Tris-HCl, pH 8.5, 50 U T4-polynucleotide kinase (Boehringer Mannheim). Incubate at 37 °C for 30 min. Dilute into 300 µl of loading buffer for "NENSORB™ 20" cartridges (NEN, DuPont), load onto cartridge, and process as described in the NENSORB manual. Precipitate with ethanol, and dissolve in 10 µl H_2O.

2. Restriction of plasmid DNA, generation of single stranded regions, annealing of 5'-end labelled primer, and sequencing reaction: 20 µl reaction volume containing 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.5 (buffer tolerates up to 100 mM NaCl if necessary for restriction), 4 µg DNA, 20-80 U (depending on compatibility with reaction buffer) of a restriction endonuclease cutting once downstream (up to 400 bp including the region to be sequenced) from the site of primer annealing. Incubate at 37 °C for 1 h, add 7 U (2 µl) T4-DNA polymerase (Pharmacia) and continue incubation at 37 °C for 30 min. Inactivate enzyme at 75 °C for 10 min. Transfer 10 µl of reaction mixture into a new vial, add 1-2 µl of the 5'-end [^{32}P]-labelled primer (>10^7 dpm), and anneal at 65 °C for 1 h. Allow to cool, add 8-10 U (2 µl) Klenow-polymerase (Pharmacia), and distribute 3 µl each into the G, A, T, and C reaction vial. Add 3 µl of the respective termination mix and continue as usual with Sanger sequencing.